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| 13. ABSTRACT (Maximum 200 Words) Mutations in the BRCA1 checkpoint gene results in aneuploidy and an increased risk of breast cancer. However, the age of onset and type of cancer can vary among BRCA1 mutation carriers and this difference is partially attributed to unlinked modifier loci. The yeast RAD9 protein has similar functions and sequence motifs as BRCA1 and we proposed to identify candidate modifier loci by identifying haploinsufficient mutations at a second locus that alters the chromosome loss rate of our rad9-/- diploid strains. To complete the screen we created a rad9-/- yeast strain carrying two different chromosome loss markers. To date, we have screened 7500 insertional mutants (25% genome coverage) for alteration in genomic stability and qualitatively identified 10 candidate insertions. By fluctuation analysis, five mutant strains have at least a ten fold increase in chromosome loss rate. The insertions include disruption of uncharacterized yeast open reading frames as well as a gene implicated in chromatin silencing. All mutants will be further characterized for alterations in radiation sensitivity, DNA damage checkpoint function and recombination. Thus, the screening strategy is capable of identifying mutations that alter genomic stability of a rad9-/- strain which can then serve as candidate modifiers for BRCA1 mutation carriers. | | | | |
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Final Report DAMD17-03-1-0464

Introduction:

The *BRCA1* breast cancer susceptibility gene was identified after a massive positional cloning effort focused on families containing at least four cases of breast cancer¹. Once the gene was identified testing for mutations became clinically available. Subsequent analysis of women who carry a *BRCA1* mutation has demonstrated that the penetrance (the likelihood one will develop cancer if you carry a mutation) is quite different depending on the population studied. Estimates of penetrance from the original Breast Cancer Linkage Consortium were as high as 80% for breast cancer. Other studies using unselected populations lead to estimates of 30-50%. Similarly, the risk for ovarian cancer is dependent on how the study is performed. One explanation for these different cancer risks is the potential for other genetic loci (termed modifier genes) to influence cancer risk.

There are several approaches to identify specific modifier loci in individuals who carry *BRCA1* mutations. The most commonly used approach is to directly test loci that influence cancer risk in the general population or have a biologically plausible reason to hypothesize an influence on *BRCA1*. For example, specific alleles of a VNTR polymorphism in *HRAS1* influences ovarian cancer risk in *BRCA1* carriers. The latter approach is exemplified by studies of genes involved in the response to DNA damage. For example, a polymorphism in the 5' UTR (untranslated region) of the *RAD51* gene influences cancer risk in women who carry *BRCA2* mutations but not *BRCA1* mutations. More recently, genome-wide studies for genetic modifiers have been initiated. The PI of this proposal is participating in a multi-institutional study through the Cancer Genetics Network to collect DNA samples and clinical data from hundreds of women who carry *BRCA1* or *BRCA2* mutations. Genome-wide scans will be used in a modified linkage approach to identify loci that influence the age of cancer diagnosis in these women. These linkage methods are likely to be successful but are very large-scale efforts that when successful still implicate a locus that may contain dozens to hundreds of genes. No straightforward methods are available to sort the genes in the region for potential to carry an important polymorphism. Therefore, in this Exploratory Award we proposed to develop an innovative approach to use the simple eukaryote, *Saccharomyces cerevisiae*, to rapidly screen the genome for mutations that modify genomic instability. The genes identified in this study can then be candidates for focused epidemiologic studies.

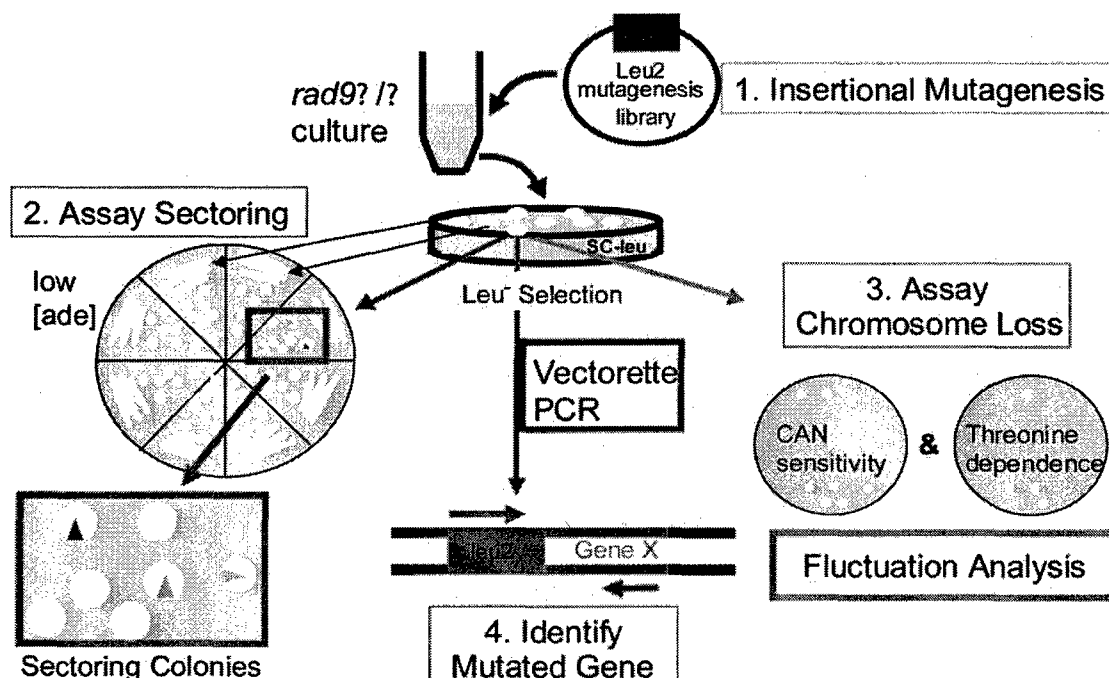
Though no direct ortholog of the human *BRCA1* gene been identified in the *S. cerevisiae* genome the human *BRCA1* protein is similar to the budding yeast Rad9 protein in several ways². *rad9* mutant strains and *BRCA1* mutant cells have defective G2 checkpoint responses and are sensitive to several forms of DNA damage including ionizing radiation, alkylating agent exposure and UV radiation. In addition, the ATM (mutated in ataxia telangiectasia) kinase phosphorylates *BRCA1* in response to DNA damage just as Mec1, a *S. cerevisiae* ATM homolog, phosphorylates Rad9 in yeast. Furthermore, both *BRCA1* and Rad9 proteins contain a C-terminal tandem repeat of the BRCT (*Brc1* carboxy terminus) motif. Mutating the Rad9 BRCT domain results in phenotypes similar to those observed in *rad9Δ* strains confirming that it is an important functional domain. Mutations in the *BRCA1* BRCT motif results in an increased risk of breast cancer in women.

As first reported by Weinert and Hartwell³, exponentially growing *rad9Δ* strains show a 10- to 20-fold increase in the spontaneous rate of chromosome loss. Recently, Klein and colleagues directly compared chromosome loss rates among a series of checkpoint defective *S. cerevisiae* strains. They found that *rad9Δ/Δ* strains had a chromosome loss rate of 2.0×10^{-5} compared with 2.0×10^{-6} for a comparable wild type strain. Based on this conservation in function, we proposed to develop and perform novel genetic screens in yeast that will allow rapid identification of loci which when haploinsufficient modify the chromosome loss phenotype (either increase or decrease) of a *rad9Δ/Δ* diploid strain. These genes will serve as candidate modifier loci of genomic instability resulting from loss of *BRCA1* function in mammary cells.

Body

In the sections below we document the progress towards completion of the tasks in the original statement of work. For each section we have included our progress towards completing that task. Figure 1 gives an overview of the strategy we developed over the course of this Exploratory Award to complete the genetic screen.

Figure 1 – Overview of the Genetic Screen Devised to Identify Heterozygous Mutations that Impact Chromosome Loss Rate of *rad9* Mutant Diploid Strains



Statement of Work:

Task 1 – Engineer *rad9Δ/Δ* strain for rapid and visual selection of mutants which alter chromosome loss rate using marked mini-chromosomes.

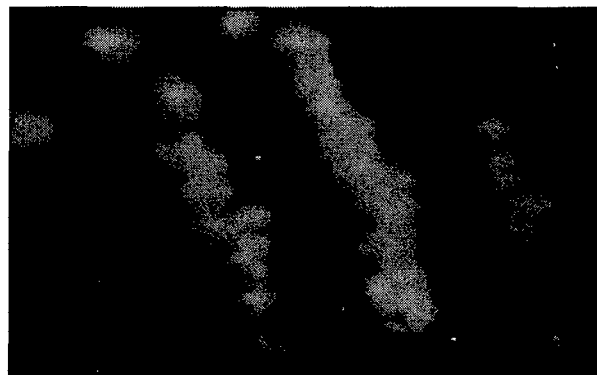
- Mutate endogenous *ADE 2* gene and introduce mini-chromosome containing *ADE2* gene in selected diploid strains.
- Optimize plating method to screen library for changes in rate of sectoring due to loss of mini-chromosomes.

- c. Optimize assays to measure rate of forming can^r , $\text{met}^{+/-}$ colonies.

Progress

Task 1a. In order to use the *ADE2* marker, we engineered *rad9Δ/Δ* diploid strain to contain an *ade2-101* mutation which is an ochre mutant that is capable of being suppressed by a *SUP11* tRNA gene contained on a chromosome fragment (CF). Dr. Vicki Lundblad kindly supplied us with yeast strains containing a CF with one copy of the *SUP11* gene. In the absence of the *SUP11* gene, strains carrying an *ade2-101* mutation will cause a yeast strain to build up adenine precursors and turn a red color. When a diploid yeast strain carries two CFs, and thus two copies of *SUP11*, the mutation and subsequent build up of precursors are suppressed and the yeast returns to a wild-type white color. However the suppression via *SUP11* is leaky and thus carrying only one copy of *SUP11* (one CF) in a diploid *ade2-101/ade2-101* background results in a pink colored yeast colony. Any genomic instability resulting in CF loss can be visualized in this system as the appearance of pink or red sectors on a white colony due to lack of suppression of the *ade2-101* mutation. We then utilized this system to visually quantitate CF loss rate by counting colonies that sector, indicating a loss or gain in the copy number of the *SUP11* gene. (See example of assay in Figure 2.)

Figure 2 – Left: Example of Streak Out of a Mutant Strain in order to Monitor for Loss of the Chromosome Fragment. Right: An Enlargement of the Area in the Green Box Demonstrating Four Red/Pink Sectorial Colonies.



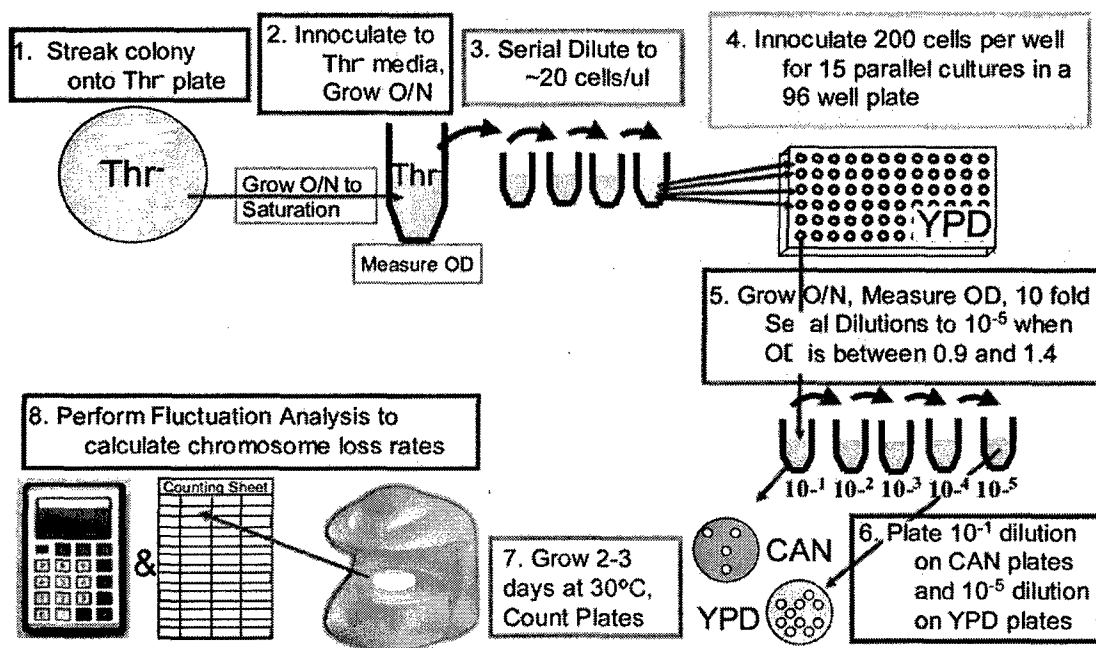
Task 1b. We carried out a number of experiments to determine the expected level of CF loss rate in the parental strain and the variance in this measurement. After a number of trials we determined that if we streak out a strain on one eighth of a 10 cm petri dish we expect to see fewer than 1 sectorial colony (out of about ~200 colonies in that area of the plate). We set this level for mutant strains to be further characterized in subsequent tasks as any mutant that had 4 or more sectors in the 1/8 streakout.

Task 1c. Dr. Hannah Klein has graciously supplied us with *rad9Δ/Δ* yeast strains carrying a doubly marked chromosome V for assaying whole chromosome loss rates⁴. Chromosome V carries the *CAN1* gene on one arm of the chromosome and *HOM3* on the

other arm. The CAN1 gene encodes the yeast arginine permease, responsible for uptake of arginine and also capable of transporting the arginine ortholog and toxic drug L-canavanine into the cell. Strains that are CAN1/can1-100 are sensitive to canavanine, but losing the wt copy of the gene confers resistance to the drug as it is no longer taken up into the cell. The HOM3 gene is involved in threonine biosynthesis and is required for growth in the absence of threonine in the media. These markers allow us to distinguish chromosome loss events by identifying cells that are canavanine resistance and unable to grow on threonine-deficient media.

The method used to measure chromosome loss rate is termed fluctuation analysis. This allows one to determine actual mutation rate (as opposed to mutation frequency, which reflects both mutation/loss rates as well as the growth of the culture). Optimizing this task in order to efficiently screen the mutant strains derived in this strain took significant work (approximately 6 months). We utilized the Spectrofluor Plus purchased by this grant in order to rapidly quantify the growth rate of the mutant strains. Figure 3 specifies the multiple steps required to determine the chromosome loss rate of the mutant strains. We have verified that this method allows us to calculate the chromosome loss rate of a given strain within a factor of 2 compared to prior published studies. In addition, the variance of measurements of the same strain done three times is also within a factor of two. Thus, this methodology is sufficient for our goal of identifying strains that have a chromosome loss rate that is about five-fold increased compared with the parental strain.

Figure 3 – Protocol Developed to Efficiently Monitor the Chromosome Loss Rate by Fluctuation Analysis of the Mutant Strains Selected by the Sectoring Assay



Task 2 – Perform insertional mutagenesis screen using Synder Library DNA into strains developed in Tasks 1 and 2.

- a. Assay for changes in chromosome loss rate.

Progress:

Task 2a: We optimized the method to use the LEU2 insertional Snyder library described in the original application. In order to screen the entire yeast genome we will need to screen approximately 30,000 LEU+ mutant colonies. We have obtained these colonies and thus far we have struck-out and analyzed ~7500 LEU+ insertional mutagenized colonies for their sectoring phenotype. Of these 7,500: 105 met our increased sectoring threshold by having >4 sectors in 1/8 of a plate streak-out (Figure 1 is an example of one of these strains). We then restruck-out these 105 colonies and identified 10 which continued to demonstrate a >4 sectoring phenotype and another 30 with a consistent intermediate phenotype. The 10 with the >4 sectoring phenotype were taken through the whole chromosome loss rate assay and analyzed via fluctuation analysis. We identified 5 mutant strains (highlighted in green in Table 1) that expressed increased whole chromosome loss rates with at least a 10 fold increase over the non-mutagenized *rad9Δ/Δ* strain. During the next year we will complete the screen of another 22,500 mutant colonies in order to effectively screen the entire yeast genome.

Table 1. Sectoring Frequencies and Whole Chromosome Loss Rates of Insertional Mutants

Task 3 – Develop high throughput method to grid insertional *LEU2* insertional mutants.

| Strain | Primary Streak Sector Number | Re-Streak Sector Number | Chromosome loss rate |
|----------------|------------------------------|-------------------------|----------------------|
| <i>rad9Δ/Δ</i> | | | 2.0×10^{-5} |
| mutant 1264 | 9 | 4 | 9.9×10^{-6} |
| mutant 1283 | 5 | 4 | 9.9×10^{-6} |
| mutant 1357 | 9 | 5 | 4.2×10^{-3} |
| mutant 1375 | 5 | 7 | 3.5×10^{-4} |
| mutant 1428 | 5 | 7 | 1.3×10^{-4} |
| mutant 1518 | 10 | 10 | 3.1×10^{-5} |
| mutant 1855 | 12 | 8 | 9.6×10^{-6} |
| mutant 1978 | 6 | 10 | 3.4×10^{-4} |
| mutant 2155 | 5 | 3 | 2.5×10^{-5} |
| mutant 2393 | 5 | 5 | 4.7×10^{-3} |
| mutant 2896 | 5 | 4 | 4.3×10^{-5} |
| mutant 3199 | 17 | 8 | 3.1×10^{-5} |

- a. Transform *rad9Δ/Δ* parental strain with Snyder Library.
- b. Grid library into 384 well plates.
- c. Develop assays to detect quantitative changes in chromosome loss rate of the gridded library. Months
- d. Screened gridded libraries using methods developed in 3c.

Progress:

Task 3a-b: We developed a high throughput method for analyzing chromosome loss rates as described in Task 2. However, this methodology still requires a 96-well methodology. Thus, we have not gridded the mutants into the 384 well plate format. We purchased with departmental funds an optical reader (AcoLyte) that allows us to count

Can^r colonies on standard petri dishes. The use of this reader (as opposed to hand counting) increases the efficiency of the chromosome loss rate by at least ten fold thus achieving the goal of having a higher throughput method for screening the insertional mutants..

Task 3c-d: See Task 2b-c.

Task 4 – Isolation of mutant locus identified in genetic screens performed in Tasks 2 & 3.

- Vectorette PCR protocol to identify locus of *LEU2* insertion.
- Creation of targeted disruption of genes flanking *LEU2* insertion in *rad9Δ/Δ* and wild type strains.
- Characterization of targeted mutant strains for genomic stability, DNA damage sensitivity, checkpoint response.

Progress:

Task 4a: We optimized the methodology to perform vectorette PCR of our *LEU2* insertional mutants. We will also verify these mutations by Southern blot analysis and a plasmid rescue assay in bacteria. Vectorette PCR on the 5 insertional mutants has been used to identify the insertion site for each mutant. These PCR fragments were sequenced. The sequence was then used to run BLAST search of the yeast genome to identify the gene into which the insertion fell. From these 5 mutants we have identified 4 separate insertion sites (see Table 2), two that fell into the *DIP5* gene (a dicarboxylic amino acid permease), one in *YLR307C-A* (uncharacterized gene), one into a non-coding region on chromosome IX (which may contain a novel gene) and one into the open reading frame of the *SIR4* chromatin remodeling protein. Thus, the vectorette PCR assay is working efficiently to allow us to identify the insertion sites derived from the mutagenesis protocol.

Table 2: Results of Vectorette PCR Analysis of Mutagenesis

| Strain | Chromosome loss rate | Transposon Insertion Site |
|--------------------|----------------------|--|
| <i>rad9-/-</i> | 2.0×10^{-5} | — |
| <i>mutant 1357</i> | 4.2×10^{-3} | <i>DIP5</i> : Dicarboxylic amino acid permease |
| <i>mutant 1375</i> | 3.5×10^{-4} | <i>SIR4</i> : Chromatin Silencing |
| <i>mutant 1428</i> | 1.3×10^{-4} | "Non-coding" region insertion site |
| <i>mutant 1978</i> | 3.4×10^{-4} | <i>YLR307C-A</i> : Uncharacterized |
| <i>mutant 2393</i> | 4.7×10^{-3} | <i>DIP5</i> : Dicarboxylic amino acid permease |

Task 4b-c: We are currently performing these aims on the five insertional mutants described above including (1) constructing deletion of the inserted genes, (2) determining whether nearby genes show increased expression and (3) determining if there are undocumented ORFs at the insertion site. To this end we have looked at the identity of nearby genes and found many involved in the actin cytoskeleton, and cell cycle genes. We plan to utilize Northern blots or semi-quantitative RT-PCR to look at the mRNA expression of these candidate genes. Also, to show that the insertion site we identified is the only insertion event that took place we will perform Southern blots with a probe to the transposon to look for the number of junction fragments. Task 4c will be conducted once the corresponding mutant strains are created.

Task 5 - Writing of manuscripts and final report.

Progress:

We have submitted an abstract for the Annual Meeting of the American Association of Cancer Research (AACR) describing our development of this genetic screen and the identification of a chromatin remodeling protein in the screen. The abstract is currently under review and it is our expectation that the abstract will be presented at the meeting in Anaheim, CA in April, 2005. As requested, we have also submitted an abstract to the DOD Era of Hope Meeting for June, 2006.

Writing of a scientific paper will await further analysis of the role of SIR4 remodeling protein in modifying chromosome loss rates. In addition, we plan over the next year (utilizing a no cost extension) to complete the screen of the *rad9Δ/Δ* mutant strain for other insertional mutants which impact chromosome loss rates and then write a paper describing the methodology for the screen and all of the mutant strains identified.

Key Research Accomplishments

Development of a *rad9Δ/Δ* mutant strain that contains two markers of chromosome loss:

- (1) a chromosome fragment containing the *sup11* tRNA gene and an endogenous chromosome V containing the *can1-100* mutation.
- (2) development of an efficient, moderately high throughput methodology to quantitate the chromosome loss rate of the tester strain using fluctuation analysis in a 96 well format.
- (3) identification of five insertional mutants that alter the chromosome loss rate of a *rad9Δ/Δ* strain.
- (4) Use of vectorette PCR methodology to identify the insertion site for each of the five mutant strains specified in (3).

Reportable Outcomes

Because this was an Exploratory Award, the bulk of the last 18 months were spent developing the methodology required to complete the screen. Thus, we are not yet at the stage where we have many reportable outcomes:

Insertional mutagenesis coupled with efficient fluctuation analysis allows identification of heterozygous mutations that impact the chromosome loss rate of a *rad9Δ/Δ* mutant strain.

Abstract under review.

Strome, E. and S.E. Plon (2005). Utilizing budding yeast to identify potential modifiers of BRCA1 function. Submitted to the American Association of Cancer Research Annual Meeting.

Conclusions

The goal of this Exploratory Award was to investigate the novel hypothesis that we could develop a methodology to identify heterozygous mutants that impact the genomic stability of a *rad9* mutant strain. We have completed this goal and have developed a protocol that allows efficient screening of the yeast genome for such mutations. With approximately 25% of the yeast genome completed we have already identified heterozygous mutations in unexpected genes, including *SIR4*, that increase the chromosome loss rate of the *rad9* mutant strains. Completion of this screen and analysis of the mutants obtained will allow us to identify a set of genes which are candidates for modifiers of BRCA1 function. Professor Timothy Rebbeck of the University of Pennsylvania has expressed interest in using these loci in his ongoing epidemiology study to identify genetic modifiers that impact that age of onset of breast cancer in women who carry mutations in BRCA1.

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